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Abstract: Yeast Saccharomyces cerevisiae strains have been constructed that co-express cDNAs coding for the human cytochrome P-450 enzymes CYP1A1 or CYP1A2 in combination with human NADPH-cytochrome P-450 reductase (oxidoreductase). Microsomal fractions prepared from the strains were able to efficiently activate various drugs to Salmonella mutagens. These experiments demonstrated that a functional interaction occurred between the respective human enzymes in the yeast microsomes. For every drug tested, the microsomes containing CYP enzymes and oxidoreductase were 2- to 4-fold better in activation than the corresponding microsomes that contained CYP alone. Interestingly, co-expression of CYP1A2 with oxidoreductase resulted in a decrease of 7-ethoxyresorufin-O-deethylase activity, a problem which is related to this specific substrate. Using the microsomes, it was demonstrated that aflatoxin B1, was activated to a mutagen not only by CYP1A2 but also by CYP1A1. In contrast, benzo[a]pyrene was exclusively activated by CYP1A1 whereas CYP1A2 was inactive. The drug 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) was activated by CYP1A2 and to a lesser extent by CYP1A1. A strong substrate specificity was observed with the two structurally related heterocyclic arylamines 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). MeIQx was activated efficiently by both CYP enzymes, whereas MeIQ was only activated by CYP1A2 and not by CYP1A1. The fact that microsomes from vector transformed control strains were unable to activate any of the drugs studied underlines the suitability of these microsomes for metabolic studies. Moreover, the presence of suitable marker genes in the yeast strains will enable us to study mitotic recombination and gene conversion events induced by drugs that require metabolic activation

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High promutagen activating capacity of yeast microsomes containing human cytochrome P-450 1A and human NADPH-cytochrome P-450 reductase

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Yeast *Saccharomyces cerevisiae* strains have been constructed that co-express cDNAs coding for the human cytochrome P-450 enzymes CYP1A1 or CYP1A2 in combination with human NADPH-cytochrome P-450 reductase (oxidoreductase). Microsomal fractions prepared from the strains were able to efficiently activate various drugs to *Salmonella* mutagens. These experiments demonstrated that a functional interaction occurred between the respective human enzymes in the yeast microsomes. For every drug tested, the microsomes containing CYP enzymes and oxidoreductase were 2- to 4-fold better in activation than the corresponding microsomes that contained CYP alone. Interestingly, co-expression of CYP1A2 with oxidoreductase resulted in a decrease of 7-ethoxyresorufin-*O*-deethylase activity, a problem which is related to this specific substrate. Using the microsomes, it was demonstrated that aflatoxin B_1 was activated to a mutagen not only by CYP1A2 but also by CYP1A1. In contrast, benzo[*a*]pyrene was exclusively activated by CYP1A1 whereas CYP1A2 was inactive. The drug 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) was activated by CYP1A2 and to a lesser extent by CYP1A1. A strong substrate specificity was observed with the two structurally related heterocyclic arylamines 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline ($MeIQ_x$). $MeIQ_x$ was activated efficiently by both CYP enzymes, whereas MeIQ was only activated by CYP1A2 and not by CYP1A1. The fact that microsomes from vector transformed control strains were unable to activate any of the drugs studied underlines the suitability of these microsomes for metabolic studies. Moreover, the presence of suitable marker genes in the yeast strains will enable us to study mitotic recombination and gene conversion events induced by drugs that require metabolic activation.

Introduction

Carcinogenesis is often accompanied by specific alterations in two important classes of genes, the proto-oncogenes and the tumor suppressor genes. The products of these genes make up a complicated molecular network involved in cell-cycle regulation and differentiation. It is not surprising that subtle disturbances in this delicate network can have profound detrimental effects like neoplasia. Mutations that arise spontaneously or are induced by environmental agents in the two classes of genes have been recognized as an important factor of carcinogenesis. Therefore, several test systems have been developed where the mutagenic effect of a given compound on various organisms from bacteria (1,2) to mouse (3,4) can be recognized.

Apart from mutations, the genetic event described as homologous mitotic recombination also plays an important role in carcinogenesis (5). Mitotic recombination provides an attractive mechanism for the loss of heterozygous tumor suppressor genes. It has indeed been recognized that heterozygous wild-type tumor suppressor gene copies are not only inactivated by further mutations, but recombination-mediated loss has clearly been demonstrated in a few cases (6,7). Such a loss of heterozygosity can simply be explained by a single crossover occurring during the G_2 phase in the homologous DNA between the gene locus and the corresponding chromosomal centromere. Chromatid exchange will lead to a segregation of the two alleles in half of the following mitotic divisions. Thereby clones are created that have become homozygous for the gene locus of interest. For this reason it is not only important to learn the mutagenic activity of a chemical compound to which humans are exposed, but a potential recombinagenic activity of the compound should also be considered in cancer risk assessments.

In contrast to a large battery of test systems designed for the identifiation of mutagens, only few systems exist where recombinagens can be detected (8–10). One of these is based on the lower eukaryotic yeast *Saccharomyces cerevisiae* and uses strain D7 which has been constructed by Zimmermann and colleagues (11). Strain D7 allows for the simultaneous phenotypic detection of mitotic recombination, gene conversion and reversion. To extend the metabolic capacity of this strain we have genetically engineered it to confer on it specifically defined human enzyme activities. For this purpose we have introduced plasmids that direct the expression of human cytochrome P-4501A cDNAs in combination with a NADPH-cytochrome P-450 reductase (oxidoreductase*) cDNA. In this study we have asked whether the newly introduced cDNAs were functionally expressed in the yeast strains. To this end we have prepared microsomal fractions from the strains and used them as alternative activation systems in the Ames test. Here we show that two important classes of carcinogens, polycyclic aromatic hydrocarbons (PAHs) and heterocyclic arylamines are efficiently activated to *Salmonella* mutagens. As a member of the first class of compounds we chose benzo[*a*]pyrene (BaP) which is activated by a combination of cytochrome P-450 (CYP) mediated epoxidation and hydrolysis by epoxide hydrolase to the ultimate carcinogen (for review, see ref 12). BaP and other PAHs are produced in combustion processes and are present as urban environmental pollutants. The other class of compounds, heterocyclic arylamines, are natural constitutents of our diet and they have been identified particularly in broiled and fried fish and meat (13). Heterocyclic arylamines are activated by hydroxylation of the amino group, which is predominantly catalyzed by cytochrome CYP1A2 to an intermediate which is subsequently acetylated by a *N*-acetyltransferase

*Abbreviations: oxidoreductase, NADPH cytochrome P-450 reductase; PAHs, polycyclic aromatic hydrocarbons; BaP, benzo[*a*]pyrene; CYP, cytochrome P-450; PMSF, phenylmethylsulfonylfluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethylketone; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; DMSO, dimethyl sulfoxide; AFB1, aflatoxin B_1; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; $MeIQ_x$, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; EROD, 7-ethoxyresorufin-*O*-deethylase.

to yield the ultimate mutagen (for review, see ref 14). Our results demonstrate that both classes of compounds are efficiently activated by the yeast microsomes. Therefore, the constructed strains represent a promising tool for the study of drug induced mitotic recombination.

Materials and methods

Chemicals

3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) was purchased from Pharmacare (Brüggen, Switzerland) and was dissolved in methanol; all other promutagens were dissolved in dimethyl sulfoxide (DMSO). Aflatoxin B_1 (AFB1) and BaP were purchased from Fluka (Buchs, Switzerland). 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline ($MeIQ_x$) were gifts from Dr P.Morgenthaler (Nestec Ltd, Vers-Chez-Les Blanc, Switzerland).

Strains and media

S.cerevisiae strain YHE2 (*MATa*/*MATα*, *ade2*-40/*ade2*-119, *trp5*-12/*trp5*-27, *ilv1*-92/*ilv1*-92, *ura5*Δ5/*ura3*Δ5) has been described (15). It was transformed according to standard methods (16) and transformants were cultured in minimal medium (17) containing 0.67% yeast nitrogen base w/o amino acids, 2% glucose and supplemented with 20 μg/ml adenine and tryptophane, 30 μg/ml isoleucine and leucine and 150 μg/ml valine. Untransformed strains were propagated in YPD medium (17). For microsomal preparations cultures were grown in synthetic complete medium lacking uracil (SD-ura) (17). Strain YNW64 (*MATa*, *ade2*-119, *trp5*, *cpr1*::*ura3*Δ5, *ilv1*-92, *ura3*Δ5) was provided by N.Wittekindt and derived from strain YES9 (18) by disrupting the *URA3* gene present in the *cpr1*::*URA3* allele. Details of the disruption will be described elsewhere.

Escherichia coli strain DH5αF' [F', *endA1*, *hsdR17*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, φ80d/*lacZ*ΔM15, Δ(*lacZYA argF*) U169] was used for plasmid constructions, transformed according to standard protocols (19) and propagated in LB medium (19) (containing 150 μg/ml ampicillin when appropriate).

Salmonella typhimurium strain TA98 (*hisD3052*, *rfa*, Δ*uvrB*, pKM101) was used for mutagenicity tests and was grown in NB medium (20).

Plasmid constructions

The constructions of plasmids pHE10 (15), pHE36 (21) and pSB229 (18) have been described. To construct plasmid pCS316, a 3.1 kb *Sal*I fragment containing the oxidoreductase expression cassette including promoter and terminator was isolated from pSB229 and inserted into the *Sal*I site of vector pUC7 (22). From there it was recovered as a *Bam*HI fragment and inserted into the *Bam*HI site of pBLSK-Not-Not. This latter plasmid was constructed from pBLSK (Strategene, La Jolla) by ligating the two phosphorylated and annealed oligonucleotides 5'TCGACAGCGGCCGCTG3' and 5'GTACCAGCGGCCGCTG3' into the plasmid's *Asp*718 and *Sal*I sites, thereby providing a second *Not*I site. The oxidoreductase expression cassette was subsequently recovered as a *Not*I fragment and inserted into the *Not*I sites in plasmid pCS289 and pCS290 in both orientations, to result in the plasmids pCS311, 312, 316 and 339. For the construction of the intermediates pCS289 and pCS290, a 2.2 kb *Sac*I fragment containing the CYP1A2 expression cassette was isolated from plasmid pHE36 (21) and inserted into the *Sac*I site of plasmid pNW144. This vector was a gift from N.Wittekindt and was derived from vector pDP34 (23) by converting the *Bam*HI site to a *Not*I site by linker addition. All restriction enzymes were used according to the manufacturer's recommendations.

Preparation of yeast microsomes

Microsomes were prepared essentially as described (15) with the following modifications: The procedure was scaled up to 6 l yeast cultures. Sonication of the spheroblasts was performed for 2 × 30 s in cell washing buffer containing 0.8 mM phenylmethylsulfonylfluoride (PMSF) as well as leupeptin, pepstatin and *N*-tosyl-L-phenylalanine chloromethylketone (TPCK) each at 4 μg/ml. Cell debris was removed by two centrifugations for 15 min at 11 000 *g* and 25 000 *g*. After the subsequent ultracentrifugation for 1 h at 102 000 *g*, the tube walls were cleaned with a paper tissue and the pellet was resuspended in 100 mM sodium pyrophosphate, 1 mM EDTA, pH 7.5, by gently grinding with a cold reagent tube. Ultracentrifugation was repeated, the pellet dissolved in 5 ml 100 mM sodium phosphate buffer, pH 7.4, and homogenized with a Dounce potter. Then aliquots were frozen in liquid nitrogen and kept at −80°C.

Enzyme activities

The determination of 7-ethoxyresorufin-*O*-deethylase (EROD) activities of intact yeast cells and of cytochrome *c* reductase activities of microsomes were done as described (18). EROD activities of microsomes were determined as described (21) using 5 μl microsomes and a substrate concentration of 3 μM.

Ames tests with yeast microsomes as activating system

An NADPH regenerating system containing 150 mM potassium phosphate buffer, pH 7.4, 0.74 mM $NADP^+$, 15 mM glucose-6-phosphate, 10 mM $MgCl_2$ and 14 units glucose-6-phosphate dehydrogenase per ml was freshly prepared. To 700 μl of the regenerating system, 100 μl of an overnight culture of strain TA98 was added, followed by the addition of 15–30 μl yeast microsomes. After gently mixing, promutagens were added in a volume of 30 μl. Preincubation was done at 37°C for 20 min, then 2 ml molten top agar (20) were added and poured on minimal plates (20) containing 150 μg/ml ampicillin. Plates were incubated for 48 h and revertant colonies were counted.

Western blots

Detection of antigenic proteins was done as described (21).

Results

Co-expression of cytochrome P-450 and oxidoreductase cDNAs in S.cerevisiae

The expression of human CYP1A1 (15), CYP1A2 (21) and the co-expression of CYP1A1 with human oxidoreductase (18) in *S.cerevisiae* strain YHE2 has previously been described. The expression plasmids used in this study are shown in Figure 1. Previous experiments have shown that co-expression with oxidoreductase resulted in a 16-fold increase in the CYP1A1 specific EROD activity compared to expression of CYP1A1 alone (Figure 2). This result prompted us to co-express human CYP1A2 with oxidoreductase. For this purpose a plasmid was constructed where the two human cDNAs coding for CYP1A2 and oxidoreductase are transcribed in yeast cells under control of the same constitutive GAPDH promoter. This plasmid (pCS311) and a similar plasmid lacking oxidoreductase (pHE36) were introduced into yeast by transformation and transformants were cultured in uracil-deficient medium to select for the plasmids.

To test whether a functional interaction occurred between the heterologous enzymes, EROD activities were determined in

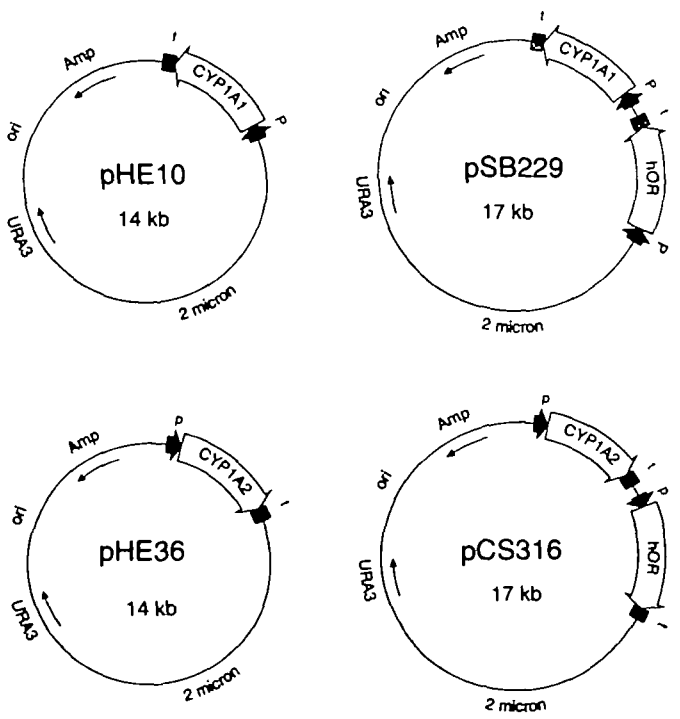

Fig. 1. Expression plasmids used in this study. The *E.coli*/*S.cerevisiae* shuttle plasmids replicate at high copy number in yeast due to the presence of 2 micron DNA. They are stable in both organisms under selection for ampicillin resistance and uracil prototrophy respectively. Individual human cDNAs (open arrows) are expressed by the *S.cerevisiae* GAPDH promoter (filled arrows denoted by p). The stippled boxes (denoted t) represent the terminator region of the *S.cerevisiae PHO5* gene. The origin of replication in *E.coli* and the oxidoreductase cDNA are denoted by ori and hOR respectively.

exponentially growing cells (Figure 2). Unlike our previous observation regarding co-expression of oxidoreductase with CYP1A1, the analogous co-expression with CYP1A2 surprisingly did not result in an increased EROD activity. In contrast, transformants containing plasmid pCS311 even exhibited less EROD activity than pHE36 transformants. Since the relative orientation of the two expression cassettes in plasmid pCS311 was different from that in pSB229, we tested whether the expression was influenced by the particular orientations of the cassettes. For this purpose the CYP1A2 and hOR expression cassettes were inserted into the vector in all four possible orientations. Nevertheless, none of these plasmids conferred a higher EROD activity than was obtained with pHE36 transformants (Figure 2).

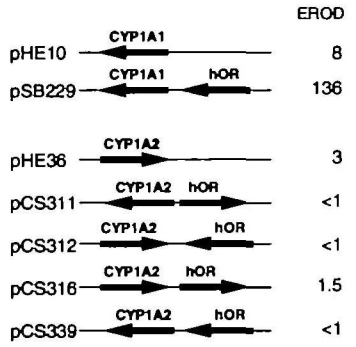

Fig. 2. CYP1A specific EROD activities of exponentially growing yeast strains. EROD activities of corresponding YHE2 transformants are given as pmol/min/3 × 10^7 cells, where 10^7 cells contain ~100 μg protein. The expression cassettes for CYP1A1, CYP1A2 and oxidoreductase (hOR) are represented by bold arrows and the orientations of the arrows give the direction of transcription. Only the relevant parts of the plasmids are shown. No activity was detectable in vector transformed controls.

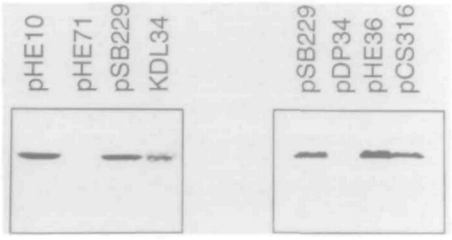

Fig. 3. Western blots demonstrating the presence of human CYP enzymes in yeast. 300 μg whole cell proteins (left) or 50 μg microsomal proteins (right) were electrophoresed on 10% polyacrylamide–SDS gels. Proteins were transferred to nitrocellulose and detected with a polyclonal antiserum directed against rat CYP enzymes from family 1 (P-450cd). CYP1A1 and CYP1A2 enzymes were present in all yeast transformants except in strains transformed with vector (pDP34) or with plasmid pHE71 (18) containing the oxidoreductase expression cassette alone. An antigenic band of the same mobility was seen in microsomes from human kidney donor liver (KDL34).

In order to discover why co-expression of oxidoreductase with CYP1A2 was unable to increase the cells' EROD activity, the presence of the heterologous enzymes in the yeast cells was tested. Crude extracts or microsomal fractions were prepared from the respective transformants and were subjected to Western blot analysis using an antiserum that recognized rat cytochrome P-450 enzymes from family 1. Figure 3 demonstrates that the strains transformed with plasmids pHE10, pSB229, pHE36 and pCS316 contained antigenic CYP proteins to a similar extent, whereas control strains lacked heterologous CYP. Evidence for the presence of human oxidoreductase in the yeast transformants was obtained indirectly. For this purpose the plasmids were transformed into the *cpr1*Δ strain YNW64 which lacked endogenous yeast oxidoreductase due to a disruption of the *CPR1* gene. Such *crp1*Δ strains are viable on normal media but they react supersensitively towards the antifungal drug ketoconazole (24). The supersensitive phenotype is rescued by expression of human oxidoreductase cDNA in such strains (18). YNW64 transformants containing individual plasmids were tested for their sensitivity towards low doses of ketoconazole. These experiments demonstrated that plasmids pSB229 and pCS316 were both able to rescue the *cpr1*Δ mutation and to confer their host strain ketoconazole resistant to a similar degree (data not shown). In addition, the >10-fold increase in cytochrome-*c*-reductase activity in pCS316 microsomes compared to pHE36 microsomes (Table I) suggested that oxidoreductase was functional. Therefore, the lack of increase in EROD activity of pCS316 transformants did not reflect a problem in the production of the two human enzymes but was presumably related to the specific substrate 7-ethoxyresorufin.

Microsomal fractions from yeast transformants activate promutagens in vitro

Batch cultures of the yeast transformants were grown and microsomal fractions were prepared. The protein content and EROD activities of the microsomes are presented in Table I. To test whether the human enzymes present in the microsomes were able to activate promutagens, various drugs were incubated with the microsomes *in vitro* and the formation of mutagenic metabolites was monitored by performing an Ames test. Table II shows that the mycotoxin and liver carcinogen AFB1 was activated to a mutagenic metabolite by microsomes containing CYP1A1 and oxidoreductase. No significant activation was observed when the microsomes or the NADPH-regenerating system were omitted from the reaction or when microsomes were used from a control strain that produced no heterologous enzymes. From this result it was concluded that human CYP1A1 which is present in the microsomes in combination with oxidoreductase

Table I. Enzymic activities of yeast microsomes

Strain	YHE2 pHE10	YHE2 pSB229	YHE2 pHE36	YHE2 pCS316	YHE2 pDP34
Human enzyme(s) produced	CYP1A1	CYP1A1 + oxidoreductase	CYP1A2	CYP1A2 + oxidoreductase	–
Protein content of microsomes (mg/ml)	3	17.2	7	8.4	11.6
EROD activity[a]	1759	3964	490	103	0
Cytochrome *c* reductase activity[b]	ND[c]	ND	167	1857	ND

[a]EROD activities are given as pmol resorufin formed per mg protein per min.
[b]Cytochrome *c* reductase activities are given as nmol cytochrome *c* reduced per mg protein per min.
[c]Not determined.

Table II. Activation of AFB1 to a mutagenic product by yeast microsomes

nmol AFB1	Yeast microsomes (516 μg) containing CYP1A1 and oxidoreductase	Yeast microsomes (348 μg) containing no human protein	NADPH regenerating system	His^+ revertants of strain TA98 per plate
15	+	−	+	1896
3	+	−	+	327
15	−	−	+	133
3	−	−	+	50
15	+	−	−	87
0	+	−	+	58
15	−	+	+	95
3	−	+	+	40
0	−	+	+	30

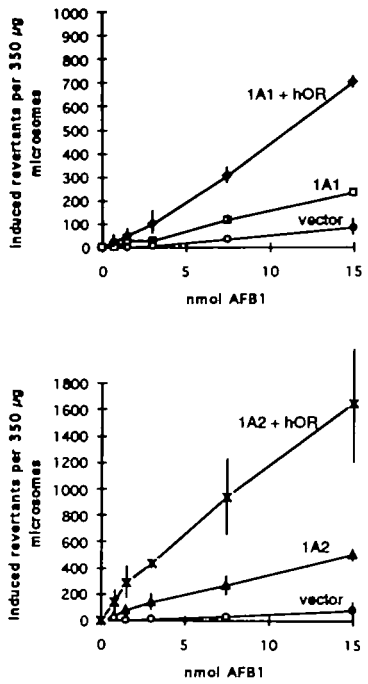

Fig. 4. Activation of AFB1 to a mutagen by yeast microsomes. ABF1 was incubated with 300−500 μg microsomal protein and His^+ revertants of strain TA98 were determined in an Ames test. The results are shown as mean values and standard deviations from two independent experiments. 1A1 and 1A2 denote microsomes from strains YHE2 pHE10 and YHE2 pHE36 that contain CYP1A1 and CYP1A2 respectively, hOR denotes the additional presence of oxidoreductase in the microsomes prepared from strains YHE2 pSB229 and YHE2 pCS316, and vector denotes microsomes from strain YHE2 pDP34 that contain no human protein.

is able to activate AFB1 to a mutagenic metabolite. A weak mutagenicity of AFB1 at the highest dose in the absence of yeast microsomes has consistently been observed and presumably reflects the contamination of our batch by a mutagenic metabolite.

In order to test whether cytochrome CYP1A1 was able to activate AFB1 in the absence of human oxidoreductase, microsomes from strain YHE2 pHE10 were used as activating system. Figure 4 shows that human CYP1A1 activated AFB1 to a mutagen which is reflected by a dose dependent increase in the observed number of revertants. The AFB1 activating potential of CYP1A1 was however markedly increased when human oxidoreductase was present in the microsomes. This result reflected the observation of increased EROD activities of the microsomes containing oxidoreductase and it argued that the

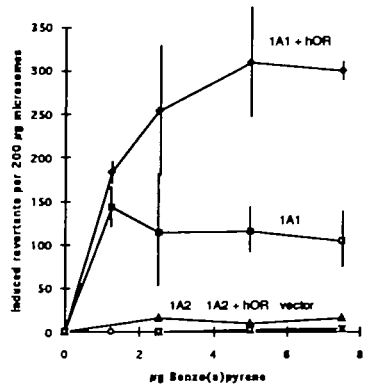

Fig. 5. Activation of BaP to a mutagen by yeast microsomes. The data points are represented as mean values and standard deviations calculated from 2−4 independent experiments. The amounts of microsomal protein in the test were 350 μg (pSB229), 60 μg (pHE10), 170 μg (pCS316), 140 μg (pHE36) and 230 μg (pDP34). The number of induced revertants were standardized to 200 μg microsomal protein. For clarity error bars were omitted in the curves for 1A2, 1A2 + hOR and vector. The standard errors for these data points were all below 22 revertants. The symbols are the same as in Figure 4.

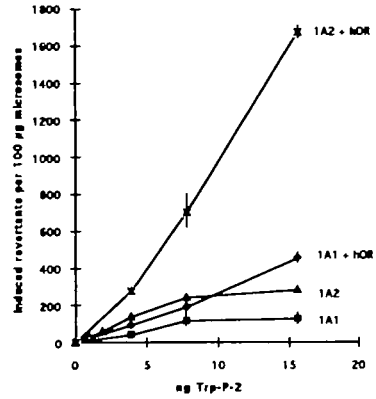

Fig. 6. Activation of Trp-P-2 to a mutagen by yeast microsomes. The amounts of microsomal proteins in the test varied between 50 and 350 μg. The number of induced revertants were standardized to 100 μg microsomal protein. Data points were calculated from double determinations. The plot represents the data from one out of 2−5 experiments performed with similar outcome. No activation was observed at the highest dose when microsomes were omitted. The number of revertants in the solvent control was 34 ± 9.

amount of endogenous yeast oxidoreductase might be limiting during heterologous cytochrome P-450 expression.

The lower part of Figure 4 shows the activation of AFB1 by human CYP1A2 enzyme. As was observed with CYP1A1, co-expression of oxidoreductase and CYP1A2 clearly increased the activating potency of CYP1A2. From this and additional results (see below) it was concluded that human oxidoreductase and human CYP1A2 present in YHE2 pCS316 microsomes indeed functionally interacted, although no increase in the EROD activity of the microsomes was detectable.

The same microsomes were used to study the activation of the PAH BaP. Figure 5 shows that YHE2 pHE10 and YHE2 pSB229 but not the other microsomes activated BaP to a mutagen. Thus, the activation of this compound could clearly be assigned to CYP1A1, whereas CYP1A2 seems to possess no activity for this substrate. Again, the presence of human oxidoreductase increased the activating potency of the heterologous CYP enzyme.

The activation of a heterocyclic aromatic amine to a mutagen by human CYP enzymes was studied with the drug Trp-P-2.

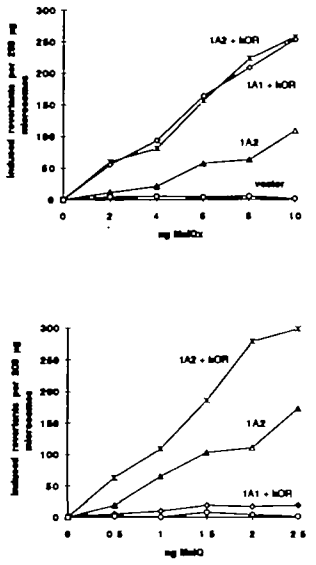

Fig. 7. Activation of MeIQ and MeIQ$_x$ to mutagens by yeast microsomes. The symbols for the data points are the same as in Figure 4. The amounts of microsomal protein in the tests were 126 μg (pCS316), 105 μg (pHE36), 516 μg (pSB229) and 348 μg (pDP34). The upper plot shows the activation of MeIQ$_x$. The data for 1A1 + hOR microsomes represent mean values of two independent experiments. The other data are from single experiments. The lower plot shows the activation of MeIQ. The data for 1A1 + hOR and 1A2 + hOR microsomes are mean values of two independent experiments. The other data are from individual experiments.

Figure 6 shows that both human enzymes CYP1A1 and CYP1A2 activated this drug to a potent mutagen. As was the case with BaP, the mutagenicity of Trp-P-2 was absolutely dependent on metabolic activation since no revertants were induced in the absence of microsomes. Cytochrome CYP1A2 was more potent than CYP1A1 in activating Trp-P-2 and the presence of oxidoreductase again increased the activating potency of both enzymes.

Two other structurally related heterocyclic aromatic amines, MeIQ and MeIQ$_x$, were tested with the same system. Figure 7 shows that CYP1A2 alone or in combination with human oxidoreductase activated both drugs to a potent mutagen. Considering the amount of drug present in the test, metabolic activation of MeIQ resulted in a slightly stronger mutagen than activation of MeIQ$_x$. A different behaviour was observed with CYP1A1. In contrast to CYP1A2 which activated both drugs, the CYP1A1 enzyme in combination with oxidoreductase exclusively activated MeIQ$_x$ to a mutagen (upper plot) whereas the same enzyme combination was unable to activate MeIQ (lower plot). This observed selective behaviour of the heterologously expressed enzymes underlines the usefulness of the yeast microsomes for studies concerning the enzymes' substrate specificities. The technical simplicity in the preparation of the microsomes circumvents the need for complex enzyme purifications.

Our experiments provided direct evidence for the functionality of the human enzymes in the yeast microsomal fractions. Moreover, the presence of the human oxidoreductase considerably increased the promutagen activating potency of the microsomes. It will therefore be interesting to see whether the drugs used are also metabolized in the intact yeast strains and whether the activated drugs will be able to induce genetic alterations other than point mutations, i.e. mitotic recombination and gene conversion. Such experiments are currently in progress and will be described elsewhere.

Discussion

This paper describes a set of yeast strains which contain human CYP1A1 and CYP1A2 enzymes that are involved in the activation of PAHs and heterocyclic aromatic amines to genotoxic compounds. In humans, *CYP1A2* is exclusively expressed in the liver (25,26). In contrast, *CYP1A1* is expressed upon induction in most other tissues, e.g. in lung and placenta of cigarette smoking individuals (27–29). Cytochrome P-450 enzymes are part of the monooxygenase complex which also contains oxidoreductase. Oxidoreductase is necessary for the electron transfer and thus for the functionality of cytochrome P-450. The lower eukaryote yeast *S.cerevisiae* contains an endogenous oxidoreductase (30) which can couple with heterologous CYP enzymes from other organisms. However, from our previous studies of heterologous CYP cDNA expression, it was concluded that the level of yeast oxidoreductase was limiting or that interaction between yeast oxidoreductase and human CYP was suboptimal (18). Here we show for both CYPs from family 1 that co-expression of human oxidoreductase significantly increases their promutagen potency. Therefore, for metabolic studies, microsomes from co-expressing strains will be superior over the microsomes from our previously constructed strains (15,21).

A similar limitation in the amount of endogenous oxidoreductase has also been observed by other groups. Kedzie and colleagues (31) reported that the activity of heterologously expressed CYP2B1 was markedly increased by the addition of purified rat oxidoreductase to the yeast microsomes. Moreover, expression of human CYP1A1 in a strain that overexpressed yeast oxidoreductase resulted in a 2-fold higher EROD activity and a 5-fold higher turnover number than without overexpression (32).

To our surprise, co-expression of CYP1A2 and oxidoreductase did not result in an increased EROD activity of the yeast culture nor of microsomal fractions thereof. However, individual tests for the presence of the two human enzymes as well as an increased promutagen activating potency clearly demonstrated that both enzymes were present and functionally interacted in the expected way. Therefore, other explanations for the lack of increased EROD activity had to be considered and a similar case described in the literature provided some evidence that the problem might be linked to the specific substrate 7-ethoxyresorufin. Resorufin ethers have been shown to be substrates not only of CYP enzymes, but also of oxidoreductase (33). The planar molecule resorufin converts to a non-planar derivative upon reduction by oxidoreductase (34). It has further been shown that the reduced form of pentoxyresorufin is not metabolized by rat CYP2B1 whereas the oxidized form is (33). In our case it might therefore be possible that the non-planar reduced form of 7-ethoxyresorufin is metabolized by CYP1A1 but not by CYP1A2. Increased levels of oxidoreductase would consequently reduce the pool of oxidized 7-ethoxyresorufin, available as substrate for CYP1A2.

By using the described yeast microsomes evidence was obtained that AFB1 was activated to a mutagen by CYP1A1 enzyme. This was particularly clear when the microsomes also contained oxidoreductase. Studies done in the past few years have shown the involvement of several CYP enzymes in the activation of AFB1 to a mutagenic and/or cytotoxic product(s). The major contribution was assigned to CYP1A2 (35–37), CYP3A4 (38, 39,40–42), CYP2A3 (35,43,44) and the activating potency was suggested to decrease in this order (45). Apart from these enzymes, minor contributions of CYP2B1 and CYP3A3 enzymes to AFB1 activation were also shown (35,46). A possible function of CYP1A1 enzyme in AFB1 activation was however less clear.

No correlation was detected between the CYP1A1 content of human liver microsomes and the mutagenicity of AFB1 incubated with the microsomes (40). Similarly, no correlation was found between the level of CYP1A1 specific mRNA and AFB1 metabolism in mouse liver (47). Furthermore, a human lymphoblastoid cell line (AHH-1) that naturally expressed CYP1A1 exhibited only a weak activation of AFB1 to a mutagen (48). However, engineering the AHH-1 cell line towards the expression of a CYP1A1 cDNA increased the cell line's sensitivity towards AFB1 by a factor of two (49). Also, a 25-fold increase in the cytotoxicity of AFB1 was observed when a monkey CYP1A1 cDNA was expressed in CHO cells which contain CYP1A1 at very low levels (50) and purified rat CYP1A1 converted AFB1 to a genotoxic product (51). Moreover, Sawada and colleagues (52) recently reported on the co-expression of mouse oxidoreductase and monkey CYP1A1 cDNAs in CHO cells observing a 9-fold higher sensitivity towards AFB1 than the parental cell line that expressed the CYP cDNA clone alone. In full agreement with their data, our data also argue that human CYP1A1 is indeed involved in AFB1 activation, and when supplemented with oxidoreductase, CYP1A1 is only twice less effective than CYP1A2.

The activation of BaP to a *Salmonella* mutagen could almost exclusively be attributed to CYP1A1 since no significant mutagenicity was obtained upon incubation with CYP1A2 containing microsomes. This result is in agreement with other reports (32,53,54). A similar weak activation of BaP by human CYP1A2 was also demonstrated by vaccinia virus expression in hepatoma cells (55).

Heterocyclic aromatic amines are known to be metabolized via a hydroxylation of the amino group (13,14) and the reaction is predominantly catalyzed by CYP1A2 enzyme (39,56). From induction studies, where rat pulmonary CYP1A1 or CYP1A2 enzymes were specifically induced, it was concluded that Trp-P-2 was also activated by CYP1A1 (57). In contrast, cDNA expression in HeLa cells of mouse CYP1A2 but not of mouse CYP1A1 provided cell lysates that activated Trp-P-2 to a mutagen. Our results clearly show that human CYP1A1 also activates Trp-P-2. It is therefore possible that the difference between mouse and human CYP1A1 with respect to Trp-P-2 activation reflects a species difference between the two enzymes. Minor discrepancies between our results and other reports concerning the activation of MeIQ and $MeIQ_x$ were also noted. Although it seemed clear that both drugs were activated by CYP1A2 (39,56,58), the involvement of CYP1A1 was more doubtful. Expression studies of human CYP cDNAs in COS cells revealed that CYP1A1 did barely activate these two drugs (58). However, from enzyme inhibition studies using rat and chick hepatocytes it was indirectly concluded that MeIQ was also activated by CYP1A1 (59). In contrast, the CYP1A1 inducer 5,6-benzoflavone did not increase the activating potency of rat liver microsomes, arguing against a role of this enzyme in MeIQ activation (57). Our results demonstrate that both heterocyclic aromatic amines MeIQ and $MeIQ_x$ are activated by human CYP1A2, but that only one of the drugs, $MeIQ_x$, is metabolized to a mutagen by CYP1A1, whereas MeIQ is clearly not. Thus, minor variations in the molecular structure of the two drugs may determine whether they are accepted as substrates by the two human enzymes or not.

In the present paper we have described a set of genetically engineered yeast strains that produce human xenobiotics metabolizing enzymes. Microsomes from the strains have been shown to activate various promutagens to mutagens. For every drug tested, the presence of human oxidoreductase has resulted in a higher activating potency compared to microsomes containing CYP alone. Therefore, co-expression of oxidoreductase might also be considered in future expression studies that aim at high activity of heterologous CYP enzymes in yeast. Moreover, the described strains might also prove useful to detect drug induced genetic alterations other than point mutations, e.g. mitotic recombination and gene conversion, which represent important mechanisms for the loss of heterozygous tumor suppressor genes, and the various marker genes present in strain YHE2 should enable us to perform such studies. Preliminary experiments have indeed demonstrated that AFB1 is metabolized by our strains to a product which significantly induces gene conversions. These results will be described elsewhere.

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